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Diode Laser-Induced Thermal Damage Evaluation on the Retina With a Liposome Dye System

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Background and Objectives: **The aim of the study was to evaluate the feasibility of retinal thermal damage assessment in a rabbit eye model by using laser-induced release of liposome-encapsulated dye.**
Study Design/Materials and Methods: **After anesthesia, thermosensitive liposomes (DiStearoyl Phosphatidyl Choline: DSPC) loaded with 5,6-carboxyfluorescein were injected intravenously to pigmented rabbits. Retinal photocoagulations were performed with a 810nm diode laser (*P*=100–400 mW, ∅=500 μm, 1s) (OcuLight®, IRIS Medical Instruments, Mountain View, CA). Fluorescence measurements in the area of the laser exposures were then realized with a digitized angiograph (CF-60UVi®, Canon-Europe, The Netherlands ; OcuLab®, Life Science Resources,® UK).**
Results: **Fluorescent spots were observed for power ranging from 100 ± 5 mW to 400 ± 5 mW. The fluorescence intensity increased linearly with the power and reached a plateau at 280 ± 5 mW. The fluorescence intensity was correlated to the maximum temperature at the center of the laser spot with a linear increase from 42 ± 3°C to 65 ± 3°C. These results are in agreement with our two previous studies with DSPC liposomes for temperature measurements in a tissue model and then in a vascular model.**
Conclusion: **This preliminary study demonstrates the possibility of a laser-induced release of liposome-encapsulated dye for a quantification of diode laser induced thermal damage in ophthalmology. Such a method could be useful for a *real-time* monitoring of laser photocoagulation for conditions such as choroidal neovascular membranes when a precise thermal damage is required near the foveolar area. Lasers Surg. Med. 24:61–68, 1999.** **© 1999 Wiley-Liss, Inc.**

Key words: fluoroescence; laser; liposomes; retina; thermal damage

INTRODUCTION

There is an increasing interest in the clinical application of diode lasers. Evidence of their usefulness for the treatment of choroidal conditions has been demonstrated by studies showing the efficacy of diode laser photocoagulation in the treatment of choroidal neovascular membranes [1]. The efficacy of diode laser has also been demonstrated for the treatment of diabetic macular edema [2]. However, some doubts have been raised as to the suitability of the infrared lasers

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for retinal photocoagulation because of difficulties for the control of the intensity of the retinal burn [3]. For central retinal pathologies, the best preservation of central visual function would require the most adequate, i.e., the minimal, dose of laser irradiation to produce the required tissue damage. However, a number of efforts to optimize the dosage of laser-induced photocoagulation has had limited or no success. Currently the usual method for ophthalmologists to control the laser-induced thermal damage remains the bare observation of blanching of the retina after a laser exposure.

Several methods have been proposed to improve the dosage of laser photocoagulation. The first one consists of estimating the time-temperature history by measuring the tissue surface temperature using infrared radiometry. Provided a real time measurement of temperature is achieved, the thermal damage can be calculated quasi-instantaneously by digitized integration using the temperature response and empirical rate coefficients of the tissue [4–8]. However, since ocular media do not transmit far infrared radiation, this method cannot currently be used in ophthalmology. The second method is based on the analysis of reflectance changes on the tissue surface during the laser exposure [9–13]. This method has characteristics that would allow an application in ophthalmology. However, it has been demonstrated that the latency time of the reflectance change could be a drawback for this method when the temperature elevation induced by the laser is low : reflectance would be a good parameter to establish a real time monitoring of laser-induced thermal damage only when the temperature reached is superior to 70°C [14].

We have already proposed a new technique of thermal damage evaluation based on the liposome-dye system. This liposome-dye system is based on the release of a fluorescent dye encapsulated in thermosensitive liposomes: previous studies were performed in vivo successively on two models using rat liver as a tissue model and thereafter on hamster skin vessels as a vascular model. At the level of the laser exposures, a release of a fluorescent dye encapsulated in liposomes was induced by the temperature elevation. The fluorescence emission intensity was correlated with temperature elevation and with thermal damage [15–18].

In addition, from these studies and those published by Khoobehi et al. [19–21] about laser triggered retinal angiographies on monkeys' eyes, it has been assumed that this liposome-dye system had the characteristics that would allow retinal photocoagulation quantification in ophthalmology: noninvasive, possible utilization through the ocular media, and low latency (<75 ms) of acquisition of fluorescence related to the temperature elevation.

This preliminary study was performed in vivo on rabbit eyes with a diode laser (810 nm). It aimed to establish the feasibility of this liposome-dye system for the control of laser-induced thermal damage in ophthalmology. The fluorescence measurements performed on this model after liposomes injection and laser irradiations were compared to the laser pulse parameters.

MATERIALS AND METHODS

Background of the Liposome-Dye System

The mechanism of the liposome-dye system is based on the properties of temperature sensitive liposomes. These liposomes are capable of existing in two distinct phases, the "gel" or "solid" phase and the "liquid crystalline" or "fluid" phase. The transition from the solid phase is defined by a transition temperature. This transition leads to the leakage of the dye entrapped in the liposomes. The phase transition temperature (PTT) corresponds to a level of maximum permeability due to the chain-melting phase transition [22,23]. However, as shown by Magin and Niesman [22,23], permeability of the liposomes membranes appears a few Celcius degrees before the PTT is reached and it remains for a few degrees after PTT. Using DSPC liposomes (DiStearoyl Phosphatidyl Choline), we have already demonstrated that the PTT is 55°C ± 3°C and that the fluorescence intensity increases linearly between 42 ± 3°C and 65°C ± 3°C [15–8]. In this liposome-dye system, neither the encapsulated dye, nor the DSPC phospholipids of the liposome membrane absorb the 810 nm infrared light, avoiding any dye release due to direct light absorption. Figure 1 shows the release characteristics as a function of temperature.

Preparation of the Dye

5,6-carboxy-fluorescein (Eastman Kodak) was used. The main excitation peak of this dye is 490 nm. The fluorescence emission is maximum at 515 nm. This dye was purified using a procedure described by Ralston et al. [24]. This purification was achieved by treating with activated charcoal, washing, and finally performing hydro-

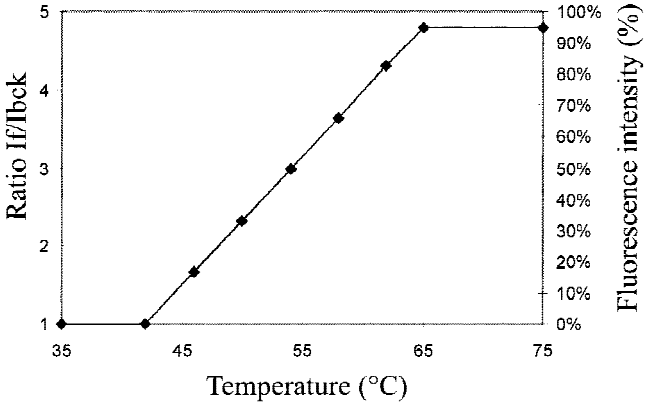

Fig. 1. Evolution of the ratio IF/Ibck as a function of temperature. T ± 3°C, Ratio ± 0.4. DSPC liposomes loaded with 5,6-Carboxyfluorescein ; laser : 810nm diode laser [from Mordon and Desmettre, 17].

phobic chromatography on an Sephadex LH-20 (Pharmacia-Biotech, Sweden) column. Fractions were analyzed by HPLC (HPLC: High Pressure Liquid Chromatography) and purified fractions were pooled together and desiccated. A 100 mM solution was prepared by dissolving a suitable amount of the purified dye in water. At this concentration there is a fluorescence quenching [25].

Preparation of Thermosensitive Liposomes

The DSPC temperature-sensitive liposomes used in this experiment are multilamellar vesicles. They were prepared by sonication, a procedure that has been described in a previous report [26]. A suitable amount of L-alpha-distearoyl phosphatidylcholine (DSPC: 18 carbon chains: liquid-crystal line phase transition temperature = 54°C, Lipoïd K.G., Ludwigshafen-Germany) was dissolved in chloroform (Merck, Darmstadt-Germany). The phospholipid organic solution was evaporated under reduced pressure in a rotary evaporation flask. After complete removal of chloroform, a 100 mM 5,6-CF solution was added and hydration of lipids was carried out at 55°C for 1 hour. After an equilibration period, the lipid suspension was sonicated at 55°C during 20 minutes under nitrogen (Sonicator Heat-System, Sonics & Materials, Danbury, CT) using the following parameters: 500W, 10% output, 20,000 Hz, 3 mm diameter probe. The liposome suspension was centrifuged at 4,000 rpm for 20 minutes (Heraus, Hanau-Germany) in order to eliminate titanium particles. Liposomes size was determined by quasielastic light scattering at a 90° angle (Sematech, SM 633/RTG, Nice-France). Mean size was determined to be 300 nm. The supernatant was dialyzed over 5 mM phosphate buffer saline (pH:7.4) for 24 hours (1,000 ml, changed twice) to remove unencapsulated 5,6-CF. Immediately after dialysis, liposomes were injected to animals to prevent any leakage of the dye.

Diode Laser

A 810 nm diode laser, OcuLight®, IRIS Medical Instruments, was adapted on a slit lamp (Takagi, Japan). This diode laser system was used with a three-mirror Goldman lens in a continuous mode. In order to increase the reproducibility of the laser irradiations the spot diameter and the pulse duration were kept constant (∅: 500 μm, pulse duration: 1 second). The laser power ranged from 100 mW to 400 mW. The power was verified with a PW-2 power meter (Synrad, Bothell, WA).

Animals

Animal procedures were performed in accordance with the ARVO statement for the Use of Animals in Ophthalmic and Vision Research. The rabbit eye was used in this study as a healthy eye model for photocoagulation without any opacities or heterogeneity of the lens. The laser pulses were performed on the midperiphery to limit focusing errors. The homogeneous pigmentation of the fundus reduced the variation of laser light absorption. Seven male pigmented rabbits weighing between 3.0–3.5 Kg were slightly anesthetized with an intramuscular injection of a combination of Ketamine (Rhône Mérieux, France) (150 mg/Kg) and Chlorpromazine (Spécia Rhône Poulenc Rorer, France) (0.50 mg/Kg). Ketamine and chlorpromazine were then injected into the marginal ear vein to ensure the anesthesia of the animal during the whole experimentation. Pupil dilatation was achieved with Tropicainamide 0.5% (MSD-Chibret) and Phenylnephrine (10%) (MSD-Chibret) eyedrops. After completing the experiments, the animals were killed with an overdose of ketamine and chlorpromazine.

Fluorescence Imaging System

A CF-60UVi® Canon-Europe® fundus camera (Amstelveen, The Netherlands) was coupled to a Kodak Megaplus 1,317 × 1,035 pixels, 8 bits monochrome camera. The output of the camera was fed to a digitizing system OcuLab®, Life Science Resources,® (Cambridge, UK) using a Pentium 133 with 32 Mb of RAM. The fundus camera was used in a 60° field mode with excitation and emission filters set for the angiography mode. The

excitation flash intensity was at maximum (F-8 corresponding to Iso sensitivity ISO = 3).

Methods

After anesthesia, a retinal image was acquired with the fundus camera (autofluorescence). The liposome solution was injected intravenously and a few images were recorded (autofluorescence after liposomes injection) just before performing the diode lasers pulses. A few minutes after the liposome injection, 3–10 laser impacts were delivered to the midperipheral region of the retina inferior to the myelinated fibers layer, one disc diameter apart. Fluorescence images of the retina were recorded immediately after the laser pulses. During the acquisition of these images the fluorescence spots were usually visible with the continuous lamp illumination between the flashes. However, the flash intensity was kept constant to increase the reproducibility of fluorescent measurements. The images were focused on the fluorescent spots to limit optical aberration that could occur during spot size measurements near the edges of the images.

Analysis of the Images

The fluorescence images were stored on magnetic disk. Their analysis provided informations about the background fluorescence, the fluorescence intensity, the size of the fluorescence spot.

For the fluorescence intensity analysis, we have used the histogram stretching procedure of the OcuLab® software that provides a representation on 256 grey levels the smaller range of grey levels of the actual image. This procedure allowed us to calculate with accuracy the ratio of fluorescence intensity (If) on the background fluorescence (Ibck). We have analyzed the size of the fluorescence spots and the evolution of the If/Ibck ratio as a function of the laser power and tissue temperature. The temperature was determined using the DSPC liposome calibration curves established on the previous models.

RESULTS

All the animals were examined with the retinal imaging system. Five fluorescence measurements were removed from the analysis because of focusing errors either when performing the laser exposures or during the imaging of the retina (image out of focus, movement of the eye, or blinking reflex). Forty-seven fluorescence measurements were included in this analysis.

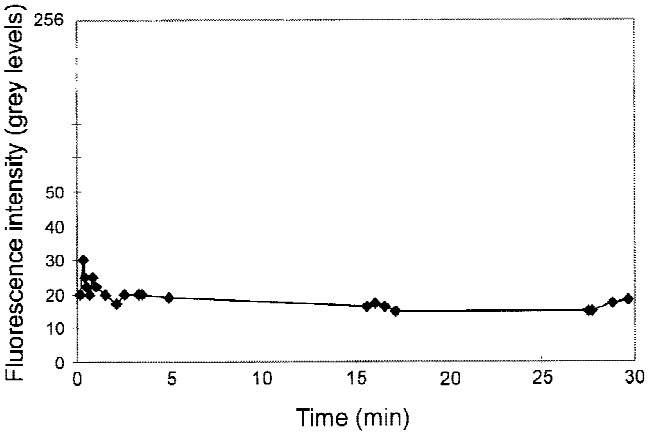

Fig. 2. Background fluorescence as a function of time after the liposome solution injection. Fluorescence intensity is displayed with the gray level scale.

Background Fluorescence

A small brief release of fluorescein leading to vessels fluorescence arose immediately after liposomes injection. After a few minutes, this brief leakage led to an homogeneous background fluorescence of the fundus. The stability of this background fluorescence provided a basis for the calculation of the ratio of fluorescence intensity (If) on the background fluorescence intensity (I_{bck}) (ratio IF/Ibck) (Fig. 2).

Size of the Fluorescence Spot

A linear enlargement of the fluorescence area was observed with increasing power (Fig. 3). The following equation gives the relation between the fluorescence spot diameter D (pixels) and the power (mW) : D = 32.3 + 0.18 power (r^2 = 0.98).

Intensity of Fluorescence

Figure 4 represents the evolution of the ratio If/Ibck and of the fluorescence intensity as a function of laser power P (mW) and as a function of tissue temperature. One can distinguish three parts into this curve: (1) before 100 ± 5 mW: no fluorescence spot can be detected following laser irradiation, and only background fluorescence is observed; (2) between 100 ± 5 mW and 280 ± 5 mW, the ratio If/Ibck and the fluorescence intensity rise linearly with the laser power. The linear regression of this part of the curve is T (°C) = 35.6 + 6.6 Ratio IF/IBck (r^2 = 0.98); (3) for power

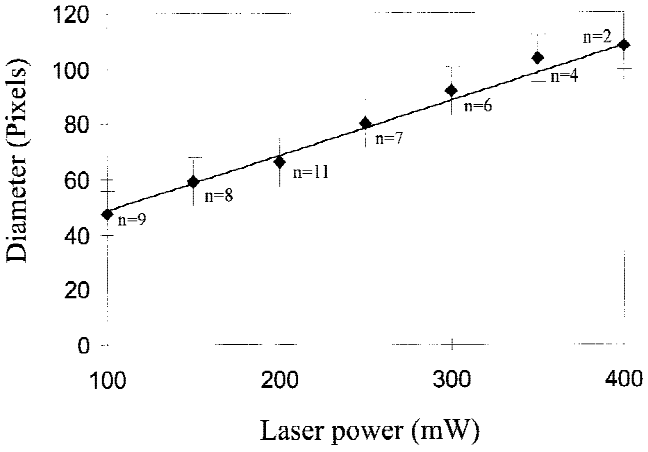

Fig. 3. Fluorescence spot diameter as a function of laser power (810 nm diode laser, $P = 100$-400 mW, $\varnothing = 500\mu$m, 1s) after previous i.v. injection of DSPC liposomes loaded with 5,6-CF. Aside each diameter the number of laser exposure has been displayed (total = 47).

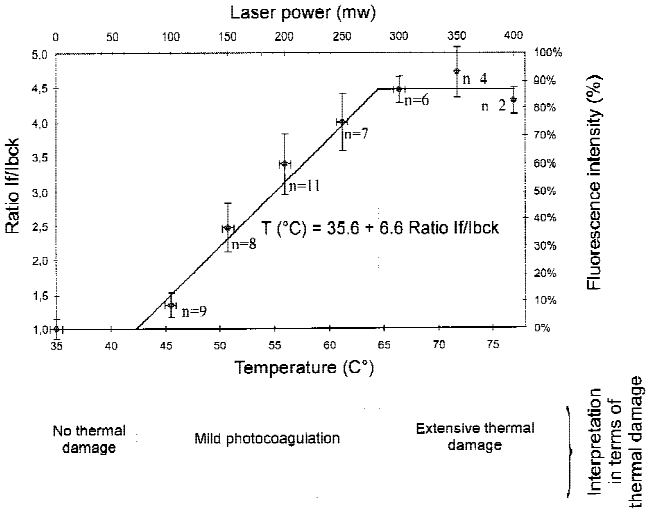

Fig. 4. Fluorescence intensity as a function of laser power and temperature. For the linear part of the curve, a linear regression has been displayed. Aside each power level the number of laser exposures has been displayed (total = 47). At the bottom of the curve an interpretation as related to thermal damage has been displayed : (1) no thermal damage zone: below 100 ± 5 mW (or below 42 ± 3°C), (2) zone with mild thermal damage: between 100 ± 5 mW and 280 ± 5 mW (or between 42 ± 3°C and 65 ± 3°C, (3) zone with extensive thermal damage : superior to 280 ± 5 mw (or 65 ± 3°C).

above 280 ± 5 mW a plateau is reached. After 350 mW the ratio IF/Ibck and the fluorescence intensity remain stable.

Retinal Fluorescence Images

Figure 5 shows one image of the fundus after the laser pulses. The laser parameters corresponding to the laser pulses have been displayed.

DISCUSSION

In this study the rabbit eye was used as a model for photocoagulation in a human eye. There was no alteration of the ocular media and no pigmentation irregularities were observed. The laser spots were administered within the mid periphery, eliminating significant discrepancy between theoretical irradiance (from the laser parameters) and real irradiance (on the retina). Focusing the laser exposures on the retina remained the main possible source of error (5 fluorescence measurements reported to focusing error have been removed from the analysis). No measurable autofluorescence occurring either from the thermal damage of the chorioretinal layers or from the liposomes could interfer with the fluorescence measurements. The range of power (100–400 mW) used in our study is in agreement with those found in the literature for infrared photocoagulation of the retina in pigmented rabbits [27–32]. Moreover the threshold radiant exposure for a chorioretinal thermal damage reported by McHugh and coworkers [32] is also in accordance with our data. In an experimental study with pigmented rabbit eyes using a diode laser (810 nm), these authors found a threshold radiant exposure of 64 J/cm^2 (P = 100mW, spot size : 200 μm, exposure duration : 200 ms) [32]. This value is close to the lowest radiant exposure (51J/cm^2 – corresponding to power = 100 mW) leading to a measurable fluorescence in our study (Fig. 4).

The fluorescence spot size curve (Fig. 3) shows the linear increase of the diameter of the fluorescence spot with increasing laser power. This linear increase corresponds to the heat diffusion from the center of the laser impact. As the heat diffuses from the laser spot, further thermosensitive liposomes are heated. The linear shape of this curve confirms on this in vivo ophthalmic model the release of fluorescein from DSPC thermosensitive liposomes induced by a temperature elevation produced by a diode laser.

The shape of the curve from Figure 4, with three parts, corresponds to those observed in previous tissue models (Fig. 1). The range of ratio IF/Ibck from 1–4.5 (Fig. 4) is also in agreement with these previous results (between 4.2 and 4.7). This fluorescence intensity curve can then be explained in the following ways. (1) Before 100 ± 5

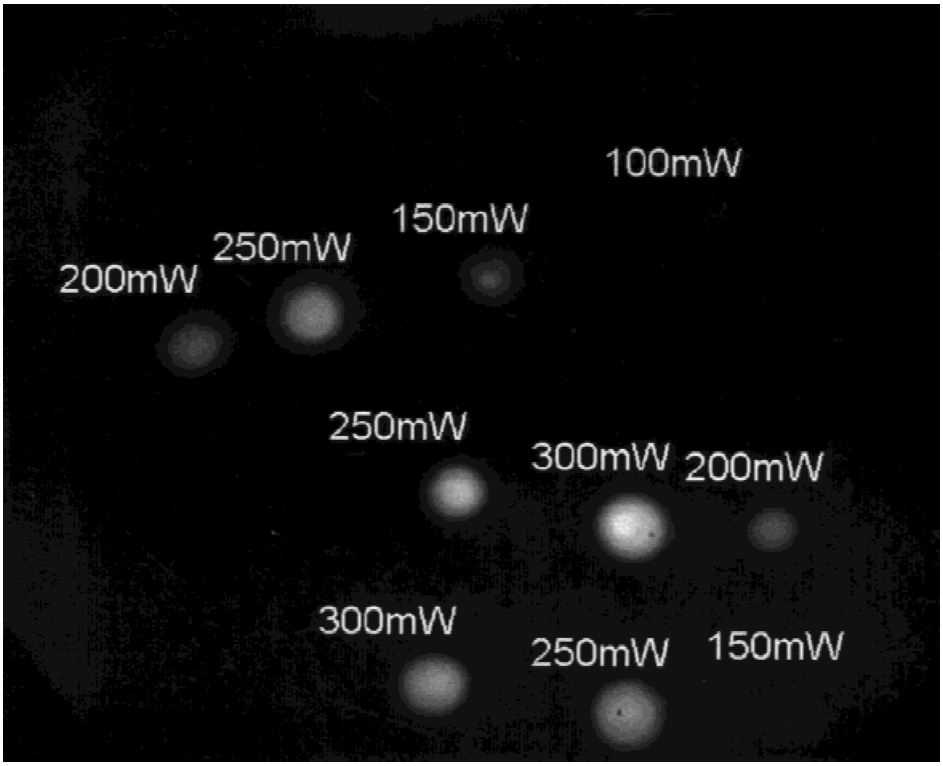

Fig. 5. Fundus fluorescent images, 4 minutes after the liposome solution injection, one minute after the laser pulses.

mW (or below 42 ± 3°C), the temperature reached is too low to induce any thermal damage and consequently a leakage of 5,6-CF from the liposomes. For this range of power the temperature is too low to destabilize the liposome. Only the background fluorescence is observed. This background fluorescence is due to some mechanical damage of liposomes during IV injection and to an interaction with blood proteins and lipoproteins occurring immediately after injection. This phenomena has already been reported in our previous studies [15–18]. (2) Between 100 ± 5 mW and 280 ± 5 mW (or between 42 ± 3°C and 65 ± 3°C), the temperature elevation induces a chorioretinal thermal damage and consequently a leakage of 5,6-CF from the liposomes. In this case, the PTT has been reached. The more elevated the temperature, the greater the amount of 5,6-CF is released from the liposomes and there is a higher measured intensity of fluorescence. This fluorescence arises from the damaged choroid and crosses the RPE and the neurosensory retina. (3) For power above 280 ± 5 mW (or temperatures above 65 ± 3°C), the fluorescence intensity reaches a plateau despite the increasing temperature. Since the maximum permeability of the liposome has been reached, the leakage of the dye is completed and consequently a further rise of temperature is not able to induce a further leakage.

Diode laser photocoagulation occurs as a consequence of the absorption of the laser light by a chromophore (principally melanin of the RPE) and conversion of light into heat. This causes a temperature elevation within the target tissue sufficient to induce protein denaturation and other local tissue changes, resulting in the ophthalmoscopically visible " laser burn. " In their histological study, McHugh and coworkers [32] found the primary site of thermal damage to be located within the retinal pigmented epithelium (RPE), and the underlying choroid. Other studies show that for infrared lasers the main zone of thermal damage is centered upon the RPE and inner choroid with some extension of tissue damage to the outer retina and mid choroid [29–31]. No evidence of diode laser-related thermal damage to vascular elements in the neurosensory retina have as yet been described [32]. These elements would claim for the inner choroid origin of the fluorescence in our model.

This feasibility study confirms that a liposome-dye system provides a reliable method to control temperature elevation within the chorioretinal layers. Consequently the possibility to as-

sess in real-time the laser-induced chorio-retinal damage is illustrated. Such an accurate control of the intensity of retinal photocoagulation requires only a digitizing system for real time fluorescence imaging.

The application of this technique in clinical ophthalmology would require consideration of the use of liposomes in humans. The safety of intravenously administered thermosensitive liposomes loaded with a fluorescent dye for an ophthalmologic use has been recently reported in two reports from Asrani and coworkers [33, 34]. The DSPC liposomes used in our experiments are composed of phospholipids among the safest for the preparation of liposomes [33–35]. Niesman [36] has recently proposed a method for stable encapsulation in temperature-sensitive liposomes of sodium fluorescein, a dye that has proven to be the agent of choice for sensitive detection of leakage of vessels of the retina. However, in our experiments, the dye loaded, 5,6-Carboxyfluorescein is similar to sodium fluorescein except for an additional carboxyl group, which makes it more water soluble and thus potentially safer as it is cleared faster from the body [37,38].

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